Federal Democratic Republic of Ethiopia

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ET ISO 9231 (2012) (English): Milk and milk products - Determination of the benzoic and sorbic acid contents

ISO INSIDE

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ETHIOPIAN
STANDARD

ES ISO :2012

First edition

Milk and milk products — Determination of the benzoic and sorbic acid contents

ICS: 67.100.01

Published by Ethiopian Standards Agency

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Foreword

This Ethiopian Standard has been prepared under the direction of the Technical Committee for Milk and Milk Products (TC 25) and published by the Ethiopian Standards Agency (ESA). The standard is identical with ISO 9231 :2008 Milk and milk products — Determination of the benzoic and sorbic acid contents published by the International Organization for Standardization ISO 2008.

For the purpose of this Ethiopian Standard, the adopted text shall be modified as follows.

- The phrase “International Standard” shall be read as “Ethiopian Standard”; and
- A full stop (.) shall substitute comma (,) as decimal marker.

Milk and milk products — Determination of the benzoic and sorbic acid contents

1 Scope

This International Standard specifies a method for the determination of the benzoic and sorbic acid contents in milk and milk products.

The method is applicable to milk, dried milk, yogurt and other fermented milks, and cheese and processed cheese, and is suitable for measuring the contents of both compounds at levels of more than 5 mg/kg.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
benzoic and sorbic acid contents
mass fractions of benzoic acid and sorbic acid determined by the procedure specified in this International Standard

NOTE The benzoic acid and sorbic acid contents are expressed in milligrams per kilogram of product.

4 Principle

Fats and proteins are removed from a slightly alkaline solution of the product by Carrez precipitation. Following dilution of the resultant solution with methanol, the supernatant liquid is filtered. The benzoic acid and sorbic acid are separated by high-performance liquid chromatography (HPLC) on a reversed-phase C_{18} column, measuring the absorbance at 227 nm and 250 nm.

5 Reagents

Unless otherwise specified, use only reagents of recognized analytical grade and distilled water or demineralized water or water of equivalent purity.

5.1 Methanol (CH_3OH).

5.2 **Precipitating reagents**, as follows:

5.2.1 Potassium hexacyanoferrate(II) solution

Dissolve 10,6 g of potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6].3H_2O$) in water in a 100 ml one-mark volumetric flask (6.3). Dilute to the mark with water and mix.

NOTE The 100 ml solution is sufficient for 40 runs.

5.2.2 Zinc acetate solution

Dissolve 21,9 g of zinc acetate dihydrate [$(CH_3COO)_2Zn.2H_2O$] and 32 ml of acetic acid (CH_3COOH) in water in a 100 ml one-mark volumetric flask (6.3). Dilute to the mark with water and mix.

If the zinc acetate dihydrate does not dissolve completely, heat the 100 ml flask and its contents in a water bath (6.2) maintained at 70 °C while swirling. When the zinc acetate dihydrate has dissolved completely, cool the solution thus obtained back to room temperature. Dilute to the mark with water and mix again.

NOTE The 100 ml solution is sufficient for 40 runs.

5.3 **Phosphate buffer solution**, pH 6,7.

Dissolve 2,5 g of potassium dihydrogen phosphate (KH_2PO_4) and 2,5 g of potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$) in 1 l of water and mix. Filter the solution thus obtained through the solvent filtration system (6.8).

5.4 **Mobile phase**, for HPLC.

Mix 10 volumes of methanol (5.1) with 90 volumes of phosphate buffer solution (5.3). Remove any dissolved gas by applying a slight vacuum.

5.5 **Sodium hydroxide solution**, $c(\mathrm{NaOH}) = 0{,}1$ mol/l.

Dissolve 4,0 g of sodium hydroxide pellets in water in a 1 000 ml one-mark volumetric flask (6.3). Dilute to the mark with water and mix.

5.6 **Sulfuric acid**, $c(\mathrm{H_2SO_4}) = 0{,}5$ mol/l.

Pour cautiously 15 ml of concentrated sulfuric acid, with a mass fraction of at least 95 % to 98 %, into 250 ml of water in a 500 ml one-mark volumetric flask (6.3) and allow to cool. Dilute to the mark with water and mix.

5.7 **Sorbic acid and benzoic acid standard solutions**, as follows:

5.7.1 Stock standard solution

Dissolve 50 mg of sorbic acid and 50 mg of benzoic acid in methanol (5.1) in a 100 ml one-mark volumetric flask (6.3). Dilute with methanol (5.1) to the mark and mix.

The stock standard solution is stable for at least three weeks if stored in a refrigerator at between 4 °C and 7 °C.

5.7.2 Working standard solution

Mix 500 ml of methanol (5.1) with 500 ml of water to obtain an aqueous-methanol solution with a volume fraction of 50 %.

On the day of use, pipette 5 ml of stock standard solution (5.7.1) into a 250 ml one-mark volumetric flask (6.3). Dilute with the 50 % aqueous-methanol solution to the mark and mix. The resulting working standard solution contains 10 µg/ml of both the sorbic and benzoic acid.

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6 Apparatus

Usual laboratory equipment and, in particular, the following:

6.1 Analytical balance, capable of weighing to the nearest 1 mg, with a readability of 0,1 mg.

6.2 Water bath, capable of maintaining a temperature of 70 °C ± 2 °C.

6.3 One-mark volumetric flasks, of capacities 100 ml, 250 ml, 500 ml and 1 000 ml, meeting the requirements for class A as specified in ISO 1042.

6.4 Liquid chromatograph, equipped with a pump capable of generating pressures of up to 4,37 MPa (6 000 psi), an injector, a dual-wavelength or diode-array UV detector, and a recorder or integrator.

The dual-wavelength detector shall have a 1 cm light path flow-through optical cell and shall be capable of measuring absorbance at 227 nm (for benzoic acid) and 250 nm (for sorbic acid).

6.5 HPLC column, made of stainless steel, of length 250 mm, of internal diameter 4 mm, containing a reversed-phase, octadecyl (ODC) treated silica adsorbent, i.e. Micro-Bondapak C_{18}1) or similar.

6.6 Syringe for HPLC.

6.7 Sample clarification kit, for membrane filtration of sample extracts, with filters of pore size 0,45 µm for aqueous solutions.

6.8 Solvent filtration system, for membrane filtration of solvents, with filters of pore size 0,45 µm for aqueous solutions.

6.9 Ultrasonic bath.

6.10 pH-meter.

7 Sampling

A representative sample should have sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707|IDF 50 [1].

Store the test sample in such a way that deterioration and change in composition are prevented.

8 Preparation of test sample

8.1 Yogurt and other fermented milks

Prior to starting the procedure, homogenize the sample by warming it gently to 40 °C while stirring. Weigh, to the nearest 0,1 g, 20 g of the homogenized sample into a 100 ml one-mark volumetric flask (6.3).

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1) Micro-Bondapak $C_{18}^{®}$ is the name of a product available commercially. This information is given for the convenience of the users of this International Standard but does not constitute an endorsement by either ISO or IDF of the product named.

8.2 Other milk products

Weigh, to the nearest 0,1 g, 3 g of sample into a 20 ml glass beaker. Disperse the test sample completely in 10 ml water added in small portions while stirring with a glass rod.

Transfer the solution quantitatively to a 100 ml one-mark volumetric flask (6.3), rinsing the beaker twice with 5 ml of water.

9 Procedure

9.1 Precipitation of fats and proteins and clarification

Add 25 ml of sodium hydroxide solution (5.5) to the test sample (see 8.1 or 8.2) and mix. Either place the flask and its contents in an ultrasonic bath (6.9) for 15 min or place the flask and its contents in a water bath (6.2) maintained at 70 °C and heat for 15 min, subsequently allowing the solution to cool to room temperature.

Adjust the pH to 8 ± 1 by adding sulfuric acid solution (5.6) while mixing. Then add 2 ml of potassium hexacyanoferrate(II) solution (5.2.1) and 2 ml of zinc acetate solution (5.2.2) to precipitate the fats and proteins. Shake vigorously and allow the suspension thus obtained to stand for 15 min. Subsequently, add about 40 ml of methanol (5.1) and mix. Cool to room temperature.

Dilute with methanol (5.1) to the mark and mix again. Allow the mixture to stand for another 15 min. Filter the supernatant liquid using the sample clarification kit (6.7).

9.2 High-performance liquid chromatography (HPLC)

Perform the HPLC analysis at room temperature, using the mobile phase (5.4) at a flow rate of about 1,2 ml/min. Allow the system to equilibrate for at least 30 min before injecting any solutions.

Then inject 5 µl to 20 µl of the clarified solution prepared in 9.1 and an equal volume of working standard solution (5.7.2). Monitor column effluent by UV detection at 227 nm and at 250 nm, using consecutive detection at the two wavelength settings or simultaneous dual-wavelength detection.

NOTE Provided that the calibration curve has been found to be linear, single-point calibration is sufficient.

The amount of methanol in the standard and the test sample solutions is much higher than that in the mobile phase, and this may influence peak shape and peak separation in certain cases. In such cases, check the peak shape and peak separation by comparing them with those obtained with injections of stock standard solution (5.7.1) in which the methanol (5.1) has been replaced by mobile phase (5.4), using equivalent volumes.

The approximate retention times for benzoic acid and sorbic acid under these conditions are 5,5 min and 7 min, respectively. If the peaks produced by either compound go off scale, prepare a suitable dilution in 50 % aqueous methanol and inject 5 µl to 20 µl of the diluted solution to obtain suitable peak heights.

To detect whether any interfering compound is co-eluting with the sorbic acid, check the ratio of the UV signals, both at 250 nm and 227 nm.

When analysing milk or dried milk, a third peak, eluting after about 8 min, is observed. This peak is produced by hippuric acid, a natural constituent. The hippuric acid peak may partly overlap that of sorbic acid. The column resolution between sorbic and hippuric acid should therefore preferably be >1.

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10 Calculation and expression of results

10.1 Calculation

Calculate the sorbic acid content, w_s, and/or the benzoic acid content, w_b, both expressed in milligrams per kilogram, using the following equation:

$$w_\mathrm{s}\left(\text{or } w_\mathrm{b}\right) = \frac{H_\mathrm{ts} \times m_\mathrm{s} \times V_1}{H_\mathrm{st} \times m \times V_2} \times 1\,000$$

where

H_ts is the peak height or area, in appropriate units, given by the test solution (see 9.2);

H_st is the peak height or area, in the same units, given by the working standard (see 9.2);

m_s is the mass, in micrograms, of working standard injected (5.7.2);

m is the mass, in grams, of the test sample (see 8.1 or 8.2);

V_1 is the volume, in millilitres, of the extract prepared in 9.1 (= 100 ml);

V_2 is the volume, in microlitres, of test solution injected (see 9.2).

10.2 Expression of results

Express the results to the nearest whole number.

11 Precision

11.1 Interlaboratory testing

The values derived from interlaboratory tests may not be applicable to content ranges and matrices other than those given.

The values of repeatability and reproducibility have been derived from the results of two interlaboratory trials carried out in 1984 and 2004 (see Annex A). The tests were carried out on samples with benzoic acid and sorbic acid contents ranging from 6 mg/kg to 920 mg/kg.

11.2 Repeatability

The absolute difference between two individual test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will be greater than $2{,}235 + 0{,}031\,\bar{w}_\mathrm{s}$ (or $\bar{w}_\mathrm{b}$) mg/kg in not more than 5 % of cases.

11.3 Reproducibility

The absolute difference between two individual test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, will be greater than $8{,}987 + 0{,}130\,\bar{w}_\mathrm{s}$ (or $\bar{w}_\mathrm{b}$) mg/kg in not more than 5 % of cases.

12 Test report

The test report shall specify:

a) all information necessary for complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

d) all operational details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the result(s);

e) the test result(s) obtained and, if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Interlaboratory trials

The values for the repeatability and reproducibility have been derived from the results of two international interlaboratory trials carried out in 1984 [4] and 2004 [6], respectively.

The results obtained in the two trials were subjected to statistical analysis in accordance with ISO 5725:1981 and ISO 5725-2:1994, respectively, to give the precision data shown in Table A.1.

Table A.1 — Results of interlaboratory trials

Parameter	Benzoic acid				Sorbic acid		
	1984 [4]	**1984 [4]**	**2004 [6]**	**2004 [6]**	**1984 [4]**	**2004 [6]**	**2004 [6]**
Samples	A [a]	B [a]	C [b]	D [b]	B+ [c]	C [b]	D [b]
Total number of laboratories	14	14	9	14	14	14	14
Outliers	2	2	1	1	3	1	0
Number of laboratories remaining	12	12	8	13	11	13	14
Mean value, mg/kg	26,4	40,6	7,61	35,0	36,1	920	57,0
Repeatability, r, mg/kg	2,0	3,8	1,6	4,4	3,4	30,5	4,5
Repeatability relative to mean, r_{rel}, %	7,7	9,4	20,5	12,7	9,4	3,3	7,8
Repeatability standard deviation, s_r, %	0,7	1,3	0,6	1,6	1,2	10,8	1,6
RSD$_r$, %	2,7	3,3	7,2	4,5	3,3	1,2	2,8
Reproducibility, R, mg/kg	13,5	15,3	11,0	9,9	12,1	128,9	18,6
Reproducibility relative to mean, R_{rel}, %	51,1	37,7	144	28,4	33,5	14,0	32,6
Reproducibility standard deviation, s_R, mg/kg	4,8	5,4	3,9	3,5	4,3	45,6	6,6
RSD$_R$, %	18,1	13,3	50,9	10,0	11,9	5,0	11,5

[a] Samples A and B: Flavoured yogurt.

[b] Samples C and D: Processed cheese (see also additional information in Reference [6]).

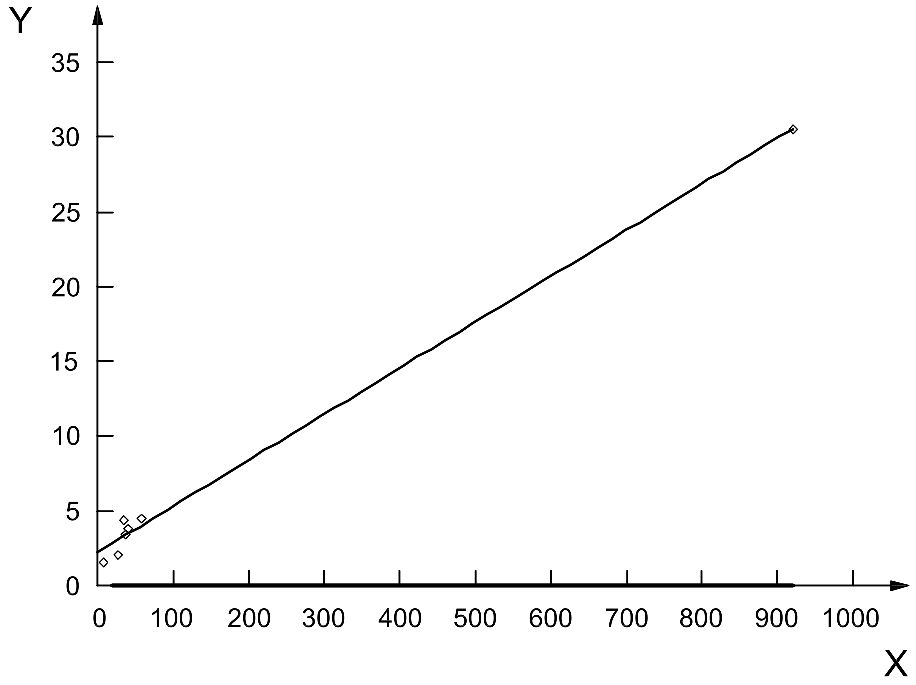
[c] Spiked with 40 mg/kg of sorbic acid during preparation.

Using linear regression analysis, the following regression line, $x = b + ay$, is obtained, with a coefficient of correlation of 0,997 348 6, for $n = 7$, $s = 0{,}824\,9$ and $t_{0,95} = 2{,}57$ (see Figure A.1):

for sorbic acid, $r = 2{,}235\,408\,8 + 0{,}030\,778\,7\ \bar{w}_{\text{s}}$ and, for benzoic acid, $r = 2{,}235\,408\,8 + 0{,}030\,778\,7\ \bar{w}_{\text{b}}$.

Using linear regression analysis, the following regression line, $x = b + ay$, is obtained, with a coefficient of correlation of 0,998 965 3, for $n = 7$, $s = 2{,}181\,5$ and $t_{0,95} = 2{,}57$ for the reproducibility (see Figure A.2):

for sorbic acid, $R = 8{,}987\,496\,5 + 0{,}130\,466\,3\ \bar{w}_{\text{s}}$ and, for benzoic acid, $R = 8{,}987\,496\,5 + 0{,}130\,466\,3\ \bar{w}_{\text{b}}$.

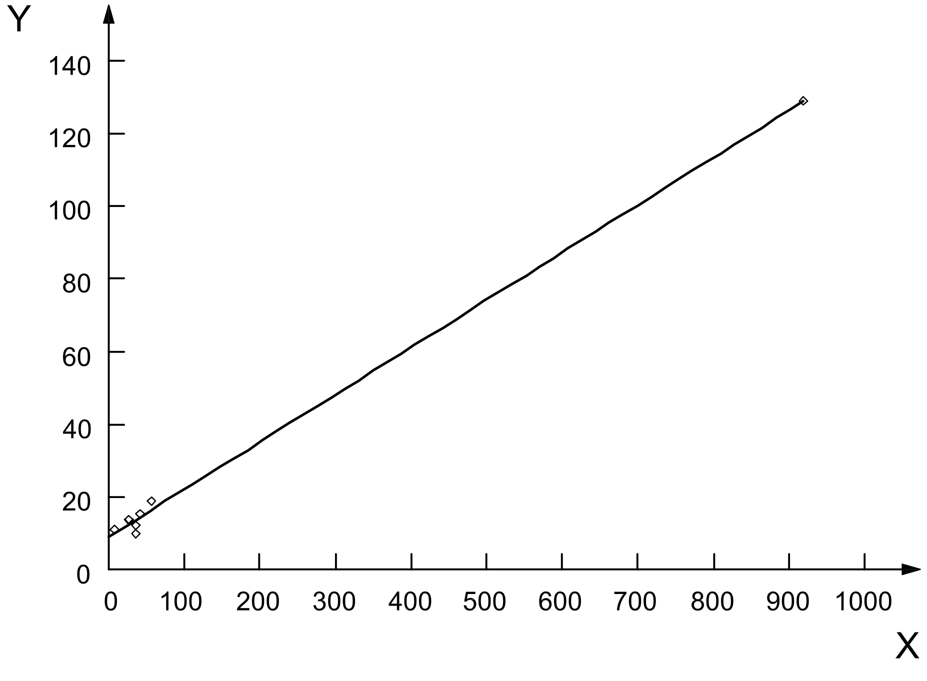


Key

X mean value (mg/kg)

Y repeatability, r (mg/kg)

Figure A.1 — Repeatability as a function of mean value



Key

X mean value (mg/kg)

Y reproducibility, R (mg/kg)

Figure A.2 — Reproducibility as a function of mean value

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Ethiopian Standards Agency

Organization and Objectives

The Ethiopian Standards Agency (ESA) is the national standards body of Ethiopia established in 2010 based on regulation No. 193/2010.ESA is established due to the restructuring of Quality and Standards Authority of Ethiopia (QSAE) which was established in 1970.

ESA's objectives are:-

- Develop Ethiopian standards and establish a system that enable to check weather goods and services are in compliance with the required standards,
- Facilitate the country's technology transfer through the use of standards,
- Develop national standards for local products and services so as to make them competitive in the international market.

Ethiopian Standards

The Ethiopian Standards are developed by national technical committees which are composed of different stakeholders consisting of educational Institutions, research institutes, government organizations, certification, inspection, and testing organizations, regulatory bodies, consumer association etc. The requirements and/ or recommendations contained in Ethiopian Standards are consensus based that reflects the interest of the TC representatives and also of comments received from the public and other sources. Ethiopian Standards are approved by the National Standardization Council and are kept under continuous review after publication and updated regularly to take account of latest scientific and technological changes.
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International Involvement
ESA, representing Ethiopia, is a member of the International Organization for Standardization (ISO), and Codex Alimentarius Commission (CODEX). It also maintains close working relations with the international Electro-technical Commission (IEC) and American Society for Testing and Materials (ASTM).It is a founding member of the African Regional Organization for standardization (ARSO).

More Information?
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